Trypanosoma brucei Mitochondrial Ribosomes

AFFINITY PURIFICATION AND COMPONENT IDENTIFICATION BY MASS SPECTROMETRY*[S]

Alena Zíková, Aswini K. Panigrahi, Rachel A. Dalley, Nathalie Acestor, Atashi Anupama, Yuko Ogata, Peter J. Myler, and Kenneth Stuart‡

Although eukaryotic mitochondrial (mt) ribosomes evolved from a putative prokaryotic ancestor their compositions vary considerably among organisms. We determined the protein composition of tandem affinity-purified *Trypanosoma brucei* mt ribosomes by mass spectrometry and identified 133 proteins of which 77 were associated with the large subunit and 56 were associated with the small subunit. Comparisons with bacterial and mammalian mt ribosomal proteins identified *T. brucei* mt homologs of L2–4, L7/12, L9, L11, L13–17, L20–24, L27–30, L33, L38, L43, L46, L47, L49, L52, S5, S6, S8, S9, S11, S15–18, S29, and S34, although the degree of conservation varied widely. Sequence characteristics of some of the component proteins indicated apparent functions in rRNA modification and processing, protein assembly, and mitochondrial metabolism implying possible additional roles for these proteins. Nevertheless most of the identified proteins have no homology outside Kinetoplastida implying very low conservation and/or a divergent function in kinetoplastid mitochondria.   *Molecular & Cellular Proteomics 7:1286–1296, 2008.*

Mt[1] ribosomes appear to be structurally variable to a considerable extent from one organism to another and have undergone some major remodeling during their evolution including loss of RNA and acquisition of proteins (1, 2). Their sedimentation coefficient (S) values vary between 50 S in *Leishmania*, 70–74 S in fungi, 55 S in metazoans, and 78 S in higher plants (3–6). Tandem mass spectrometry has been successfully applied to the analysis of the protein composition of ribosomes from several organelles including bovine mitochondria (5, 7–10), fungal mitochondria (11, 12), and chloroplasts (13, 14). Mammalian ribosomes have about the same mass as bacterial ribosomes but half as much RNA and over twice as much proteins. Proteomics analysis of bovine mt

ribosomes (9, 10) identified 78 different proteins, which include homologs of *Escherichia coli* ribosomal proteins and 36 additional proteins that presumably were acquired during eukaryotic evolution. Many of these new ribosomal proteins are distinct, having no closely related homologs in bacterial or eukaryotic cytoplasmic ribosomes. The estimated number of proteins within mammalian mt ribosomes has ranged from about 85 to more than 100, for yeast mitochondria it is 78 proteins, and for chloroplast mitochondria it is 49 proteins. The feature of protein richness implies that the reduced rRNA sizes are compensated by some protein components that might have been recruited to mt ribosomes during the course of evolution (15).

Trypanosoma and *Leishmania* are protozoan parasites belonging to the order Kinetoplastida that are the causative agents of several devastating tropical diseases such as African sleeping sickness, Chagas disease, and leishmaniasis. Protein synthesis in the mitochondria of these unicellular flagellates has unusual features. The transcripts from most genes encoded in the trypanosomatid mt DNA are post-transcriptionally modified via extensive and precise insertion and deletion of uridylates into otherwise encrypted transcripts (16, 17). The translation of the edited mRNAs was shown directly by sequencing their corresponding proteins, and surprisingly the synthesis of mitochondrially proteins was resistant to chloramphenicol (18–20). The trypanosome 9 and 12 S mt rRNAs are the smallest homologs of the *E. coli* 16 and 23 S rRNAs. The 610-nucleotide 9 S RNA has a minimal predicted secondary structure in which all four domains of the *E. coli* 16 S structure are preserved; however, some stem-loops have been greatly reduced or eliminated entirely (21). The 1150-nucleotide 12 S RNA is also greatly reduced due to the absence of domain II and some stem-loop structures in domains I and III. Domain V is the best conserved region most likely because of its functional importance as a peptidyltransferase region (22–24). The trypanosomatid rRNAs are even smaller than those present in mammalian mitochondria (12 S in the SSU and 16 S in the LSU) (25), which may imply that the trypanosomatid ribosomes may be more protein-rich. Recently the putative 40 S LSU and 30 S SSU and the 50 S monosome particles were biochemically purified from *Leishmania tarentolae* and visualized by cryoelectron microscopy, and some of the components were identified by mass spectrometry analysis (3). Interestingly an additional 45 S particle that contains only 9 S rRNA was described and designated as

[1] The abbreviations used are: mt, mitochondrial; TAP, tandem affinity purification; rRNA, ribosomal RNA; MRP, mitochondrial ribosomal protein; mAb, monoclonal antibody; SSU, small subunit; LSU, large subunit; HP, hypothetical protein; TEV, tobacco etch virus; BLAST, Basic Local Alignment Search Tool; AA, amino acid(s); PPR, pentatricopeptide repeat.

the 45 S SSU* ribosomal-related complex. This complex contains up to 49 polypeptides including several homologs of small subunit ribosomal proteins (26).

In this study we purified the mt ribosomes from *T. brucei* by tandem affinity purification (TAP) using 12 TAP-tagged component proteins, and the tagged complexes were analyzed by LC-MS/MS. We identified 133 proteins of which 38 proteins have varying degree of sequence homology to bacterial and/or mt ribosomal proteins and 56 proteins have no significant homology outside Kinetoplastida suggesting that they have diverged extensively or have unique functions in Kinetoplastida mitochondria. Based on association with rRNAs, known mt ribosomal proteins, and bioinformatics analysis, we suggest reannotation from "hypothetical" to "mitochondrial ribosomal protein (MRP)" for 29 proteins. Our results provide insight into the unique features of trypanosomatid mt ribosomes.

<div align="center">EXPERIMENTAL PROCEDURES</div>

T. brucei Transgenic Cell Lines—*T. brucei* cell (procyclic form) strains 29.13 and 1.7a were grown *in vitro* at 27 °C in SDM-79 medium containing hemin (7.5 mg/ml) and 10% fetal bovine serum.

To create the vectors for inducible expression of C-terminally tagged proteins (for candidate selection criteria see "Results") the ORFs were PCR-amplified from strain 427 genomic DNA without the termination codon (supplemental Table 1). The PCR products were cloned into pGEM-T easy vector (Promega), digested with BamHI or BglII and HindIII enzymes and ligated into the pLEW79-MHT vector (27, 28). The constructs were verified by sequencing. Genes used in this study were: TAPLSU1, Tb927.3.5610 (50 S L3); TAPLSU2, Tb927.4.1070 (50 S L13); TAPLSU3, Tb11.01.2340 (hypothetical protein (HP)); TAPLSU4, Tb927.5.4120 (HP); TAPLSU5, Tb10.70.7960 (HP); TAPLSU6, Tb10.70.7650 (HP); TAPSSU1, Tb10.406.0510 (HP); TAPSSU2, Tb09.211.2580 (30 S S17); TAPSSU3, Tb10.70.0530 (HP); TAPSSU4, Tb927.4.3690 (HP); TAPSSU5, Tb927.5.1510 (HP); and TAPSSU6, Tb927.6.2080 (HP). The TAP plasmids were linearized with NotI enzyme and transfected to 29.13 cells that co-expresses the Tet repressor protein and T7 RNA polymerase (29).

Tandem Affinity Purification of Ribosome Subunits—The TAP protocol was adapted from published methods (28, 30–32). Tagged large and small subunits were purified by sequential steps of IgG affinity chromatography, glycerol gradient sedimentation, and calmodulin affinity chromatography. Briefly cleared cell lysate was incubated with IgG-Sepharose beads, and the bound complexes were eluted by TEV protease cleavage and fractionated on a 10–30% glycerol gradient for 5 h at 38,000 rpm at 4 °C in an SW-40 rotor (31). The sedimentation profiles of the tagged complexes were monitored by Western blot analyses using anti-His$_6$ mAb. The peak reactive fractions were then pooled and further purified by calmodulin affinity chromatography, and the bound complexes were eluted with EGTA.

SDS-PAGE and Western Blotting—The protein samples were fractionated by SDS-PAGE, blotted onto PVDF membrane, probed with anti-His$_6$ mAb (1:2000; Invitrogen), and developed using an ECL system (Roche Applied Science).

RNA Dot Blot Analysis—RNA was isolated from 200 μl of glycerol gradient fractions on which mitochondrial lysate (33) or TEV eluates of TAPLSU1 and TAP_SSU2 were fractionated using TRIzol LS (Sigma) as described by the manufacturer. Isolated RNA was applied directly on a Hybond™-N+ membrane (Amersham Biosciences) using a Bio-Dot Microfiltration Apparatus (Bio-Rad). Radiolabeled oligodeoxynucleotides for 12 S rRNA (5'-GGTACATATAGAACAACTGT) and

9 S rRNA (5'-CCGCAACGGCTGGCATCC) were used as probes. Signal was visualized using GE Healthcare PhosphorImager screens, and autoradiograms were analyzed by densitometry.

Immunofluorescence Assay—Subcellular localization of the expressed tagged proteins within the cell was determined by immunofluorescence assay using anti-Myc mAb (Invitrogen). Briefly the cells were fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100, blocked with 5% fetal bovine serum, and incubated with anti-Myc antibody at 1:100 dilution. After washing, the cells were incubated with anti-mouse FITC-conjugated antibody (1:250 dilution) (Sigma), washed, and treated with 4',6-diamidino-2-phenylindole stain to visualize DNA. Phase-contrast images of the cells and their fluorescence were captured with a Nikon fluorescence microscope equipped with a camera and appropriate filters.

Mass Spectrometry Analysis—We prepared and analyzed the samples by two complementary methods. (i) The proteins were fractionated on a short length SDS-PAGE gel, and the peptides were extracted from the gel lane, and (ii) the protein mixtures were digested in solution and analyzed separately. In more details, the proteins were identified by LC-MS/MS analysis using a linear trap quadrupole (LTQ) mass spectrometer connected to a Surveyor HPLC system (Thermo Electron). The proteins in the gel pieces were digested with sequencing grade modified trypsin (Promega) overnight; the resulting peptides were extracted with 50% acetonitrile, 5% formic acid solution and dried in a SpeedVac. The TAP tag-purified protein samples were precipitated with 6 volumes of acetone, and the proteins were denatured with 8 M urea, 1 mM DTT; diluted 1:7; and digested in solution with trypsin. The resulting peptides were purified using C$_{18}$ beads (Magnetic Dynabeads RPC18, Invitrogen). The peptides from gel pieces or complex mixtures were fractionated by nanoflow liquid chromatography using a 10-cm-long × 75-μm-inner diameter C$_{18}$ capillary column and analyzed on line by electrospray ionization tandem mass spectrometry. The bound peptides were eluted from the C$_{18}$ column at a 200 nl/min flow rate with a 45-min linear gradient of 5–40% acetonitrile in 0.4% acetic acid followed by a 5-min linear gradient of 40–80% acetonitrile in 0.4% acetic acid. Xcalibur 1.4 SR1 version software was used to collect mass spectrometry data, and the mass range for the MS survey scan was *m/z* 400–1400. The MS/MS data of the five most intense ions were collected sequentially following each MS scan using the dynamic exclusion parameter where a specific ion was sequenced only twice and was excluded from the list for 45 s. The peak lists were generated using the SEQUEST module of Bioworks 3.1, cluster version SR1 (Thermo Electron), using the default parameters (molecular weight range, 400–3500; precursor mass tolerance, 1.4; group scan, 25; and minimum ion count, 15). The acquired data were compared with *T. brucei* v4.0 protein database (GeneDB) using the TurboSEQUEST program in the BioworksBrowser 3.1 software package (Thermo Electron). The database contained 9210 *T. brucei* nuclearly encoded protein sequences of which 612 are annotated as hypothetical-unlikely (34) plus 18 mitochondrially encoded protein sequences. Also mouse immunoglobulin heavy and light chains, bovine serum albumin, and human keratin sequences were included in the database. No enzyme was specified during the SEQUEST search, peptide mass tolerance was set at 1.5 Da and fragment ion tolerance was set at 0.0. No fixed modification was set for any of the amino acids, but differential modification for "Met" was set at 15.994 Da. The SEQUEST search results were filtered and compiled using PeptideProphet and ProteinProphet programs (35, 36). The data set presented here includes only the doubly tryptic peptides that have a minimum peptide identification probability of 0.9 and have a minimum SEQUEST X-correlation value of 1.5 for +1 ions, 1.8 for +2 ions, and 2.5 for +3 ions. We excluded any peptide containing more than one missed trypsin cleavage site in the sequence and that containing a cysteine amino acid because the alky-
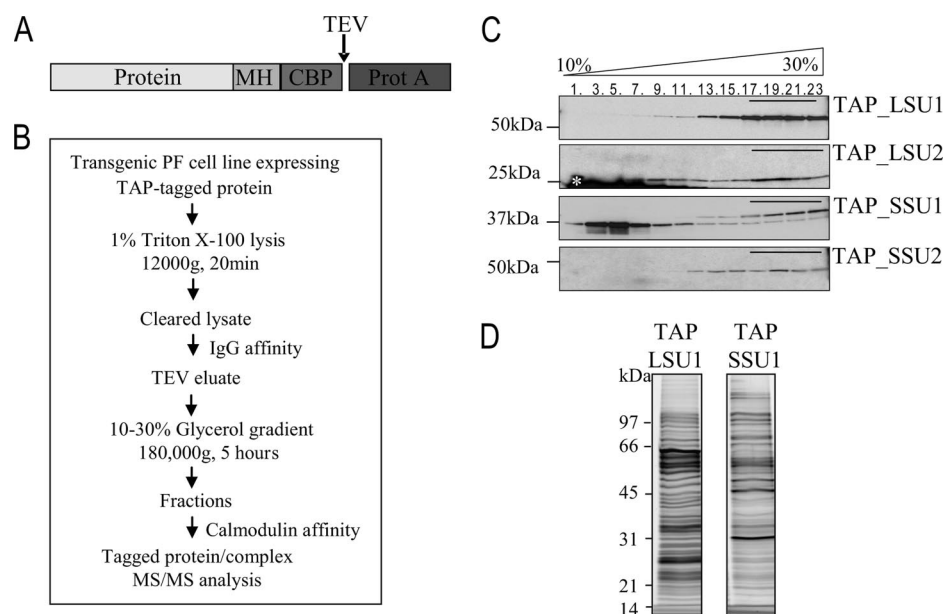
FIG. 1. **Tandem affinity purification of mt ribosomes from *T. brucei*.** *A*, scheme of C-terminal tag composed of His$_6$ and 2× c-Myc tags (*MH*), calmodulin-binding protein (*CBP*), TEV protease cleavage site, and protein A (*Prot A*). *B*, *Trypanosoma* LSU and SSU were purified using tandem affinity purification, and purified proteins were identified in the proteolytic digest followed by direct analysis using LC-MS/MS. *C*, fractionation of TAP_LSU1, TAP_LSU2, TAP_SSU1, and TAP_SSU2 TEV eluates on 10–30% glycerol gradients. Fractions were collected from the top of the gradients. Aliquots from odd fractions were analyzed by SDS-PAGE and probed with anti-His mAb. Positive fractions from the lower part of the gradient designated by a *black line* were further subjected to the second affinity purification step. The *white star* indicates the position of 25-kDa His-tagged TEV protease. The size of the protein marker is indicated on the *left*. *D*, purified TAP_LSU1 and TAP_SSU1 complexes separated on a 10–14% polyacrylamide Tris-glycine gel and stained with SYPRO Ruby. The sizes of the protein marker are indicated. *PF*, procyclic form.

lation step was not carried out during sample preparation. Proteins containing a minimum of two unique peptides matching the above criteria and with a minimum identification probability of 0.97 were considered positive.

Sequence Analysis—Bacterial and mt mammalian ribosomal proteins sequences were downloaded from the Ribosomal Protein Gene Database, and a BLAST homology search was carried out against *T. brucei* gene database (GeneDB) to identify putative ribosomal proteins. The general characteristics of the purified proteins were predicted by database homology and domain/motif searches. BLASTp and PSI-BLAST searches were carried out against the National Center for Biotechnology Information (NCBI) database to identify any homologous or related proteins. PFAM, PROSITE, InterPro, and Conserved Domain Database were used to assign motifs and domains. Probability of mitochondrial targeting sequence was predicted by MitoProt.

RESULTS

Purification of mt Ribosomes by TAP Tagging Known Subunits—The mt ribosomes from *T. brucei* cells (procyclic forms) were isolated under native conditions using the TAP tag strategy (Fig. 1, *A* and *B*). Initially two proteins of the large subunit, 50 S L3 (Tb927.3.5610) and 50 S L13 (Tb927.4.1070), that have 26 and 25% identity to L3 and L13 *E. coli* ribosomal proteins, respectively, were analyzed resulting in TAP_LSU1 and TAP_LSU2 cell lines. Similarly two other proteins of the small subunit, 30 S S11 (Tb10.406.0510) and 30 S S17 (Tb09.211.2580), that have 36 and 35% identity to S11 and S17 *E. coli* ribosomal proteins, respectively, resulted in

TAP_SSU1 and TAP_SSU2 cell lines. The 30 S S11 protein is currently annotated as a hypothetical protein in the GeneDB database. The tagged LSU and SSU were purified from cell lysate using IgG affinity chromatography, and bound complexes were released by TEV cleavage. TEV eluates were further fractionated on 10–30% glycerol gradients. All four tagged proteins sedimented at the lower part of the gradient (~40–80 S region) as determined by Western blot analysis using anti-His mAb (Fig. 1*C*) indicating their association with large protein complexes. Both TAP_LSU1 and TAP_LSU2 had a similar sedimentation profile peaking in fractions 17–21. The sedimentation peak of TAP_SSU1 and TAP_SSU2 differed slightly (19–23 *versus* 17–21), which may be due to the effect of the tag on composition of the complex. The lower band detected in glycerol gradient fractions of TAP_SSU1 is likely a degradation product of the tagged protein that is not integrated into the complex and primarily sediments at the top of the gradient. The tagged complexes were further purified from pooled gradient fractions by a second affinity purification step. SDS-PAGE analysis of TAP_LSU1 and TAP_SSU1 calmodulin column eluates showed a protein profile with numerous proteins varying in sizes from 10 to >100 kDa (Fig. 1*D*).

The sedimentation profiles of tagged and native ribosomes were compared by hybridizing 12 and 9 S rRNA probes with RNA that was isolated from glycerol gradient fractions of tagged complexes and mitochondrial extract (Fig. 2). The 12
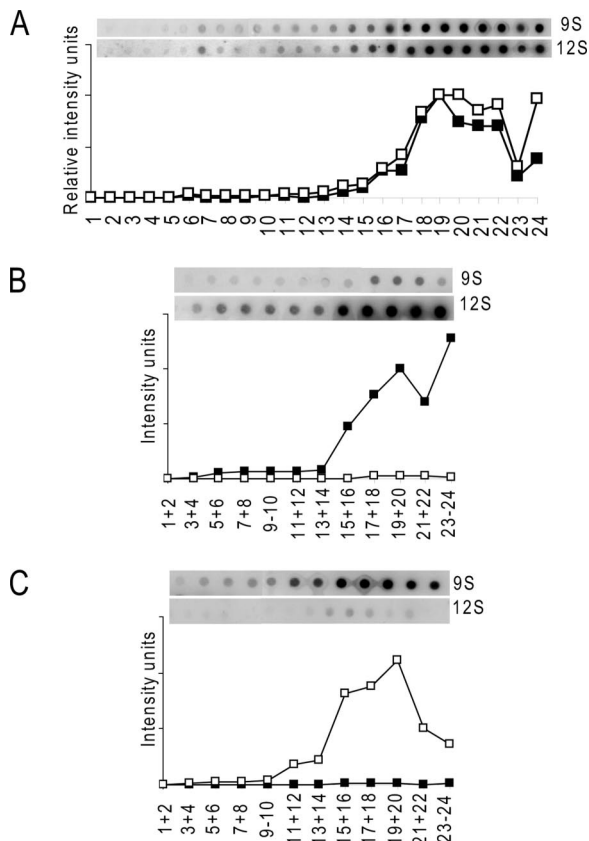
FIG. 3. **Glycerol gradient sedimentation profile of TAP_LSU3–6 and TAP_SSU3–6 TEV eluates.** Fractions were collected from the top of the gradients. Aliquots from odd fractions were analyzed by SDS-PAGE and probed with anti-His mAb. Positive fractions from the lower part of the gradient designated by a *black line* were further subjected to the second affinity purification step. The *white star* indicates the position of 25-kDa His-tagged TEV protease. The size of the protein marker is indicated on the *left*.

FIG. 2. **Glycerol gradient sedimentation analysis of mt ribosomes.** Mitochondrial extract (*A*) and TAP_LSU1 (*B*) and TAP_SSU2 (*C*) TEV eluates were assayed with probes for 9 S (*open squares*) and 12 S rRNAs (*closed squares*). The *upper panels* show the dot blot hybridization results for each fraction. These are quantified below with relative intensity units plotted on the *vertical axis* and gradient fractions plotted on the *horizontal axis*. Sedimentation is from *left* to *right*.

and 9 S rRNA from mitochondrial lysate co-sedimented in two peaks (Fig. 2*A*). One peak was centered in fractions 19–20, which corresponds to the sedimentation of the separate LSU and SSU (Fig. 2, *B* and *C*). The second peak was near the bottom of the gradient, and substantial signal in the gradient pellet likely corresponds to monosome and/or ribosome polysomes or aggregates. The detection of only 12 S rRNA with TAP_LSU1 and only 9 S rRNA with TAP_SSU1, both in fractions 19 + 20, indicates that tagged dissociated large and small ribosomal subunits were purified, and both sediment at ~60 S. A pilot LC-MS/MS analysis of calmodulin elution fractions identified 99 proteins that were present in both TAP_LSU1 and TAP_LSU2 complexes and 95 proteins in TAP_SSU1 and TAP_SSU2 complexes. These two sets of proteins were distinct and specific for large and small subunits, respectively.

Validating the Association of Novel Components—To further validate the association of newly identified proteins with ribosome subunits we tagged and analyzed four proteins of unknown function from each group. The composition of LSU
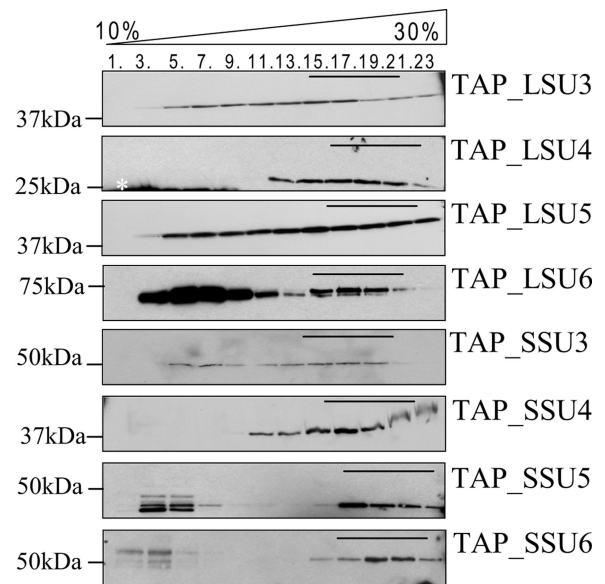
and SSU was studied in more detail using these transgenic cell lines labeled as TAP_LSU3–6 and TAP_SSU3–6 (see "Experimental Procedures" for genes). All tagged complexes sedimented in the lower parts of the gradient, although their sedimentation patterns differed, which may reflect the effect of the tag on incorporation and compactness of the complex (Fig. 3). The lower bands detected in glycerol gradient fractions of TAP_LSU6 and TAP_SSU5 are likely tagged proteins and/or their degradation products, which are not integrated into the complex and primarily sediment at the top of the gradient. The tagged complexes were further purified from pooled gradient fractions (usually fractions 15–21 or 17–23) by calmodulin chromatography.

Immunolocalization of Tagged Proteins—An immunofluorescence assay using anti-Myc mAb (anti-tag) showed that the TAP_LSU1 and TAP_SSU1 and some of the other tagged proteins (TAP_LSU3, TAP_LSU4, and TAP_SSU3) localized to the mitochondrion (Fig. 4). Together the results suggest that the TAP tags did not interfere with mitochondrial import or ribosome assembly and thus enabled us to purify mt ribosomal subunits and identify their components.

Mass Spectrometry Analysis of Tagged Ribosome Subunits—The protein composition of the tagged complexes was determined by LC-MS/MS analysis of calmodulin elution fractions. The results were compiled to assign the proteins to LSU and SSU. Based on identification of proteins in different tagged complexes they were classified into three categories. The first category (high confidence hits) includes proteins that
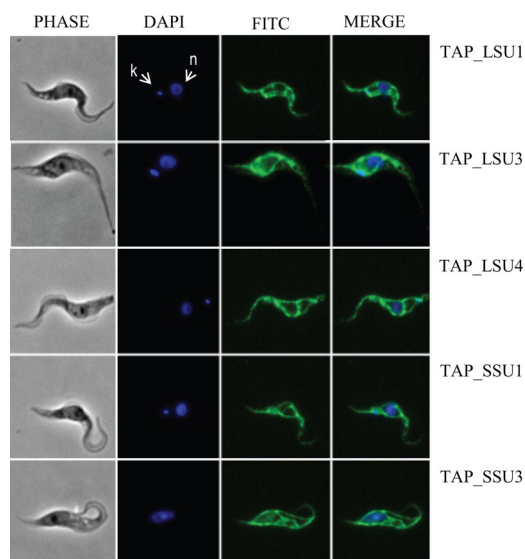
FIG. 4. **Subcellular localization of the tagged proteins.** Tagged ribosomal proteins TAP_LSU1, -3, and -4 and TAP_SSU1 and -3 were visualized in a cell using anti-Myc antibody coupled with FITC and fluorescence microscopy. Phase-contrast light microscopy shows procyclic *T. brucei* cells, 4′,6-diamidino-2-phenylindole (*DAPI*) staining is specific to DNA contents (*n*, nucleus; *k*, kinetoplast), and FITC-conjugated secondary antibody shows mt localization of the target proteins.

were identified at least in three different TAP tags for LSU or in four different TAP tags for SSU and by at least two unique peptide matches to a protein with probability of 1. Using this criteria we assigned 77 proteins as the components of LSU and 56 proteins as the components of SSU. These proteins are considered by us to be *bona fide* components (total 133 proteins) of mt ribosomes (supplemental Table 2). The second category contains proteins that were identified using the same conditions as mentioned above, but they are known subunits of different mitochondrial complexes and/or are known to be abundant mitochondrial and non-mitochondrial proteins. These proteins were operationally regarded as contaminants and were excluded from our high confidence protein list (supplemental Table 3). The third category includes proteins identified by at least two unique peptides but in less than three different TAP tags for LSU or less than four different TAP tags for SSU (supplemental Table 4).

To exclude any proteins that bind adventitiously to the common tag on each of the "bait" proteins we performed a TAP tag control involving expression of tag alone followed by its purification. MS analysis of the calmodulin eluate fraction revealed nine proteins (two heat shock proteins, prohibitin, glutamate dehydrogenase, fatty acyl-CoA synthetase 3, 60 S ribosomal protein L4, mt carrier protein, and two hypothetical proteins). Two of those proteins (60 S ribosomal protein L4 and heat shock protein) were identified in SSU complexes, and they were excluded from the high confidence list and are listed in supplemental Table 3.

Sequence Characteristics of mt Ribosomal Proteins—Most of the 133 mt ribosome proteins are currently annotated as hypothetical proteins in GeneDB database (supplemental Table 2). We performed a PSI-BLAST search against the non-redundant ("nr") database and Conserved Domain Database and PFAM, PROSITE, and InterPro domain searches and found varying degrees of similarities to other proteins of known functions or motifs for 77 proteins of which 29 proteins show a homology to known mt or bacterial ribosomal proteins (supplemental Table 2). To assess the validity of the protein assignments as ribosomal, a targeted BLAST search was carried out using *E. coli* (S1–S22 and L1–L33) and human mt ribosomal protein sequences (MRPS2–MRPS36 and MRPL1–MRPL56) against the *T. brucei* protein database and the proteins identified in TAP tag-purified samples (Table I). Although in general human MRP proteins showed homology with hypothetical proteins and annotated putative 30 and 50 S subunits, in some cases the bacterial ribosomal proteins resembled putative *T. brucei* 40 and 60 S cytoplasmic ribosomal proteins. To further verify the correct assignments of the latter proteins as cytoplasmic ribosomal proteins we used human cytosolic ribosomal proteins to search the *T. brucei* protein database. All these were also recognized by their corresponding human cytosolic counterparts with a much higher level of sequence similarity indicating that these proteins are cytosolic ribosomal protein and not mt ribosomal proteins (data not shown).

A targeted BLAST search of human 39 S mt ribosomal proteins and bacterial LSU ribosomal proteins identified 30 proteins with sequence similarities in the *T. brucei* genome, 20 of which have *E*-values less than the $1e-03$ (Table I). Only three proteins from this analysis (L10, MRPL39, and MRPL40) were not identified in our tagged complexes, and the remaining 27 proteins are present in our high confidence list. Most of these proteins are annotated as hypothetical, but because of their homology to bacterial and human mt ribosomal proteins and *in vivo* association with 12 S rRNA and other predicted mt ribosomal proteins, we propose reannotating these proteins as *T. brucei* mitochondrial ribosomal protein of large subunit x (TbMRPLx) where the "x" corresponds to the numeric designation of these proteins in bacterial and human mt ribosomal proteins (Table I).

Our analysis also corrects the annotation for some other genes. Tb927.7.4550, which is currently annotated as 60 S-like ribosomal protein, will be reannotated as MRPL7/L12. This is supported by its detection in tagged LSU and higher sequence homology to human mitochondrial MRPL7/L12 (*E*-value $7e-14$) than to 60 S cytoplasmic RPLP1 (*E*-value $2e-04$). Two *T. brucei* homologs (Tb927.3.5610 and Tb927.4.1800) of human MRPL3 and bacterial L3 protein are currently annotated as putative mitochondrial L3 proteins. Because the former is in our high confidence list and the latter was identified only in one TAP tag experiment (supplemental Table 4), we examined their homology to human 60 S ribosomal

TABLE I

List of T. brucei proteins that share the similarity to bacterial and/or mammalian ribosomal proteins
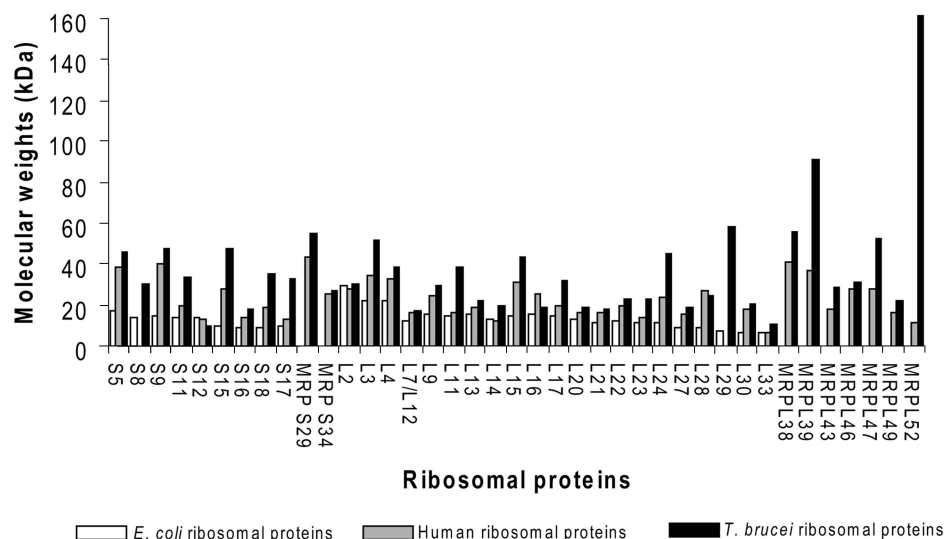
++, *E*-value is less than 1e−03; +, *E*-value is more than 1e−03; √, protein identified by MS analysis in purified ribosomal complex; *, proteins identified in 45 S SSU*-related ribosomal complex in *L. tarentolae* (26); superscript TAP_LSU1,2,6 and TAP_SSU1–3, proteins used as a bait in this study; tRNAsyn, tRNA synthetase. Dashes (—) indicate that the protein was not identified by MS analysis in purified ribosomal complex.

E. coli	Human	*T. brucei*	*E*-value	TAP tag	GeneDB	New name
L2	MRPL2	Tb927.5.3360	++	√	50 S L2	TbMRPL2
L3	MRPL3	Tb927.3.5610[TAP_LSU1]	++	√	50 S L3	TbMRPL3
		Tb927.4.1800	+	—	HP	60 S L3
L4	MRPL4	Tb11.02.3810	++	√	HP	TbMRPL4
L7/L12	MRPL7/12	Tb927.7.4550	++	√	60 S-like	TbMRPL7/12
L9	MRPL9	Tb927.5.3410	+	√	HP	TbMRPL9
L10	MRPL10	Tb11.01.3520	+	—	HP	—
L11	MRPL11	Tb927.2.4890	++	√	L11	TbMRPL11
		Tb927.2.4740	++	—	L11	TbMRPL11
L13	MRPL13	Tb927.4.1070[TAP_LSU2]	++	√	50 S L13	TbMRPL13
L14	MRPL14	Tb927.4.930	++	√	50 S L14	TbMRPL14
L15	MRPL15	Tb927.5.3980	++	√	HP	TbMRPL15
L16	MRPL16	Tb927.7.3960	++	√	50 S L16	TbMRPL16
L17	MRPL17	Tb927.8.5860	++	√	50 S L17	TbMRPL17
L20	MRPL20	Tb11.01.1930	++	√	HP	TbMRPL20
L21	MRPL21	Tb927.7.4140	++	√	HP	TbMRPL21
L22	MRPL22	Tb927.7.2760	++	√	HP	TbMRPL22
L23	MRPL23	Tb11.03.0260	++	√	HP	TbMRPL23
L24	MRPL24	Tb927.3.1710	++	√	HP	TbMRPL24
L27	MRPL27	Tb11.02.1110	+	√	HP	TbMRPL27
L28	MRPL28	Tb927.6.4040	+	√	HP	TbMRPL28
L29	—	Tb10.70.7650[TAP_LSU6]	+	√	HP	TbMRPL29
L30	MRPL30	Tb09.211.0230	+	√	HP	TbMRPL30
L33	MRPL33	Tb927.4.1810	+	√	HP	TbMRPL33
	MRPL38	Tb11.01.6620	+	√	HP	TbMRPL38
	MRPL39	Tb927.5.1090	++	—	tRNAsyn	—
	MRPL40	Tb927.5.1120	+	—	HP	—
	MRPL43	Tb927.4.4600	+	√	HP	TbMRPL43
	MRPL46	Tb927.7.4710	++	√	HP	TbMRPL46
	MRPL47	Tb09.160.5240	++	√	HP	TbMRPL47
	MRPL49	Tb927.5.3110	+	√	HP	TbMRPL49
	MRPL52	Tb11.02.2250	+	√	HP	TbMRPL52
S5	MRPS5	Tb10.70.0530*[TAP_SSU3]	++	√	HP	TbMRPS5
S6	MRPS6	Tb10.70.4850	+	√	HP	TbMRPS6
S8	—	Tb10.389.0130*	+	√	30 S S8	TbMRPS8
S9	MRPS9	Tb927.8.3110*	+	√	HP	TbMRPS9
S11	MRPS11	Tb10.406.0510*[TAP_SSU1]	++	√	HP	TbMRPS11
S15	MRPS15	Tb927.1.1200*	+	√	HP	TbMRPS15
S16	MRPS16	Tb11.02.5670	+	√	HP	TbMRPS16
S17	MRPS17	Tb09.211.2580*[TAP_SSU2]	++	√	30 S S17	TbMRPS17
S18	MRPS18	Tb10.6k15.3900*	+	√	HP	TbMRPS18
	MRPS29	Tb927.6.1250*	++	√	HP	TbMRPS29
	MRPS34	Tb927.8.5280	+	√	HP	TbMRPS34

proteins using a BLAST search. Interestingly 60 S L3 proteins strongly resemble Tb927.4.1800 (*E*-value 3e−136), but no hits were obtained for Tb927.3.5610. Based on this result we conclude that actual mt ribosomal L3 protein is Tb927.3.5610, and Tb927.4.1800 is cytosolic 60 S L3 protein. Thus the annotation of these two proteins will be changed accordingly. Ribosomal protein L11 is encoded by two identical ORFs (Tb927.2.4890 and Tb927.2.4740); thus for both of these ORFs we propose to change annotation from ribosomal L11 to MRPL11.

Using human 28 S mt ribosomal proteins and bacterial SSU ribosomal proteins we identified 11 homologs of small subunit ribosomal proteins in the genome of *T. brucei*, and all of them are present in our high confidence list. Identification of S5, S17, and MRPS29 is strongly supported by highly significant *E*-values (Table I). Although the BLAST *E*-scores for the putative S9 and S11 were not significant (*E*-values 0.99 and 0.045), an NCBI conserved domain search revealed significant homology to the RpsI family representing ribosomal pro-

tein S9 (*E*-value 3*e*−07) and conserved domain RpsK representing ribosomal protein S11 (*E*-value 0.003), respectively. Also the identification of presumed S6, S15, S16, and S18 homologs was not strongly supported (*E*-values 1.0, 0.94, 0.038, and 0.002). Using a BLAST search against the nr database the putative S15, S16, and S18 homologs are also moderately similar to *Theileria annulata* S15 (*E*-value 0.25), yeast MRPS16 (*E*-value 6.9), and *Magnetococcus* S18 (*E*-value 2.6). It is noteworthy, that *L. tarentolae* orthologs of these proteins were also identified within the purified 45 S SSU* complexes, and their homology to S6, S15, S16, and S18 mt ribosomal proteins has been reported (26). Thus, based on bioinformatics studies and the fact that these proteins are associated with 9 S rRNA we suggest changing their annotations from hypothetical to *T. brucei* mitochondrial ribosomal protein of small subunit x (TbMRPSx) where the x corresponds to the numeric designation of these proteins in bacterial and human mt ribosomal proteins (Table I).

Homologs of the bacterial large subunit proteins L1, L5, L6, L18, L19, L25, L31, and L32 and small subunit proteins S1–S4, S7, S10, S13, S14, and S19–S21 have not been found in the genome of *T. brucei*. These are either absent from the *Trypanosoma* mitochondrial ribosome, have been replaced by other proteins, or are so highly divergent that their relationship cannot be identified with confidence. The only one mt ribosomal protein, S12, which is predicted to be encoded by mitochondrial genome, was not identified in our analyses. This small protein is only 53 amino acids (AA) long and is generally interlocked into the rRNA and may not have been digested adequately during the trypsin treatment to allow its detection.

Most of the identified proteins were highly basic (pI > 9), like their homologs in other organisms, reflecting their interaction with the ribosomal RNA (5, 6, 11). Mitochondrial ribosomal proteins are significantly longer than their bacterial counterparts (7, 8, 37, 38), which probably structurally and/or

functionally compensates for the missing stem-loop structures of rRNAs. In *Trypanosoma*, all homologs of ribosomal proteins are much longer than their bacterial and human counterparts (Fig. 5). Based on the alignments, these differences in the length are usually because of N- and C-terminal extensions. One of the most striking differences is in the length of putative *Trypanosoma* MRP52 (Tb11.02.2250) that encodes for a 1522-AA protein. Only its very C-terminal part is homologous to its human counterparts MRP52-1 and -2, which have only 36 and 77 AA, respectively. The only exception to this rule is mitochondrially encoded ribosomal protein S12, which is actually smaller than its bacterial and human ortholog (Fig. 5).

Novel Proteins Associated with mt Ribosome—Some of the polypeptides identified in this work did not show homology to bacterial and/or human mt ribosomal proteins but showed homology to different proteins outside Kinetoplastida (Tables II and III). We identified several proteins with varying degrees of homology to prokaryotic and eukaryotic GTPase and/or GTP-binding proteins, methyltransferases, tRNA/rRNA methylases, pseudouridylate synthases, and helicases. It is noteworthy that two of the methylases (Tb09.211.3800 and Tb11.02.2710) are similar to putative yeast mt ribosomal proteins Ydr115wp (*E*-value 0.002) and Rsm22 (*E*-value 1.7*e*−05), respectively. Also motifs involved in protein-protein and protein-RNA interactions such as pentatricopeptide repeat (PPR), non-catalytic Rhodanese homology domain, a ubiquitin-like protein, and tetratricopeptide repeat were found within several protein sequences. Some other proteins possess the cyclophilin ABH-like domain, DnaJ motif, and domains of unknown function as such as DUF167, -390, and -436. Interestingly two proteins are predicted to be involved in amino acid and lipid metabolism (Tb927.3.970 and Tb11.01.1910). The first one also has a weak homology to yeast mt ribosomal protein MRPS35 (*E*-values 0.002). Two other proteins are putative enzymes (Tb11.01.3710 and

TABLE II

List of T. brucei proteins found in tagged LSU complexes that show some homology and/or recognizable motif to other proteins outside Kinetoplastida or are unique for Kinetoplastida mitochondria (last row)

Superscript TAP_LSU3–5, proteins used as a bait in this study; tRNAsynt, tRNA synthetase; GTP bind, GPT binding; PPIase, peptidyl-prolyl cis-trans isomerase; PseudoU_synth, pseudouridine synthase; TPR, tetratricopeptide repeat; *C. elegans*, *Caenorhabditis elegans*.

Gene ID	GeneDB	Homology search (*E*-score)	Domain search (*E*-score)
Tb927.1.1160	HP	*Arabidopsis* PPR protein (4e−06)	PPR (0.66)
Tb927.4.2720	HP	Bacterial DEAD/DEAH helicase (9e−08)	Helicase (5e−07); Helicase C (0.0084)
Tb927.6.2480	Chaperone	Heat shock protein DnaJ (3e−10)	DnaJ (1e−13); DnaJ (9.2e−24)
Tb927.6.3600	HP	Bacterial methyltransferase (1e−07)	Methyltransferase (9e−13)
Tb927.7.1640	GTP bind	Bacterial GTP binding (3e−52)	EngA2 GTPase (2e−41); MMR-HSR1 (2.2e−28)
Tb927.7.2630	HP	Bacterial GTP binding (6e−14)	YihA_EngB GTPase (1e−36); MMR-HSR1 (2.5e−21)
Tb927.7.3430	PPIase	*Arabidopsis* peptidyl-prolyl cis-trans isomerase (4e−37)	Cyclophilin ABH_like (2e−49)
Tb927.8.2760	HP	Bacterial GTP binding (5e−10)	YihA_EngB GTPase (1e−30); GTPase (9e−11)
Tb927.8.3170	HP	*Arabidopsis* PPR protein (0.001)	PPR (0.00035)
Tb09.160.2000	HP	Bacterial LSU pseudouridine synthase C (7e−25)	PseudoU_synth (3e−28)
Tb09.211.0810	GTP bind	Yeast mitochondrial GTPase (7e−21)	YlqF GTPase (2e−39); MMR-HSR1 (9.7e−05)
Tb09.211.3800	HP	Bacterial tRNA/rRNA methylase (2e−11)	SpoU-methylase (2e−07)
Tb10.70.0780	tRNAsynt	Bacterial LSU pseudouridine synthase B (1e−20)	S4 domain (0.0079)
Tb11.01.1215	PPIase	*C. elegans* cyclophilin (2e−25)	Proisomerase (5.3e−44)
Tb11.01.8760	HP	*Rickettsia* GTP binding protein (8e−15)	GTPase (2e−18)
Tb11.02.3800	HP	*Homo* pseudouridylate synthase (4e−06)	PseudoU_synth (3e−13); DUF167 (0.26)
Tb09.211.4580	HP		Lipase_3 (0.009)
Tb927.6.3930	HP		TPR-2 (0.00047)
Tb927.6.4200	HP		DUF390 (0.08)
Tb927.7.3460	HP		TPR-2 (1.9)
Tb927.7.6800	HP		eRF1 2 (0.25)
Tb10.389.1710	HP		TPR (0.32)
Tb10.70.7960[TAP_LSU5]	HP		PPR (0.00013)
Tb11.01.7140	HP		HTH-12 (0.035)
Tb11.02.3670	HP		L36 (0.56)

Tb927.3.820, Tb927.4.4610, Tb927.5.2070, Tb927.5.3870, Tb927.5.4120[TAP_LSU4], Tb927.6.1440, Tb927.6.4080, Tb927.7.2990, Tb927.7.3510, Tb927.7.7010, Tb927.8.1880, Tb927.8.3300, Tb09.160.2250, Tb10.26.0600, Tb10.26.1000, Tb10.6k15.3290, Tb10.70.5970, Tb11.01.0500, Tb11.01.1600, Tb11.01.1810, Tb11.01.1840, Tb11.01.2340[TAP_LSU3], Tb11.01.3500, Tb11.02.3230, Tb11.55.0016

Tb927.4.3690), an ADP-ribosylglycohydrolase and a superoxide dismutase (*E*-values 0.002 and 1e−08, respectively) (Tables II and III). Surprisingly 56 proteins identified in mt ribosomes (42%) have no recognizable motifs/domains and are unique to kinetoplastids (Tables II and III).

Most of the identified proteins had a predicted mitochondrial localization signal by MitoProt with a probability of more than 0.5 (supplemental Table 2). However, for some of the proteins including MRPL4, MRPL47, MRPL20, TAP_LSU4, and TAP_SSU5 the predicted probability was lower than 0.50. These results suggest diverse mitochondrial targeting signals in Kinetoplastida with some of these remaining unrecognized.

DISCUSSION

An efficient affinity purification technique coupled with mass spectrometry analysis was used to characterize the protein composition of mt ribosomes from *T. brucei*. Although these ribosomes have some compositional similarities to bacterial ribosomal proteins and/or to those of higher eukaryote they also contain proteins that have no significant homology outside Kinetoplastida as well as other proteins with various putative functions. A total of 133 proteins were consistently associated with the purified ribosomal subunits. Most of these proteins are likely to be integral LSU and SSU components. However, some of these proteins, as implied by their motifs, may have roles in other biological processes, and possibly the purified complexes are not necessarily only the conventional ribosome but may be a part of a larger "supercomplex" with additional functions.

In this study the ribosomes were purified by tagging 12 different component proteins followed by sequential steps with a first affinity chromatography, glycerol gradient sedimentation, and a second affinity purification. All of the 12 tagged proteins sedimented at 40–80 S in glycerol gradients. The sedimentation profiles differed slightly between the tagged proteins, which may reflect the effect of the tag on protein-protein or protein-RNA interactions and/or ribosome compactness. 12 and 9 S rRNAs co-sedimented in glycerol gradients with the tagged LSU and SSU, respectively.

List of T. brucei proteins found in tagged SSU complexes that show some homology and/or recognizable motif to other proteins outside Kinetoplastida or are unique for Kinetoplastida mitochondria (last row)

Superscript TAP_SSU4–6, proteins used as a bait in this study; *, proteins identified in 45 S SSU*-related ribosomal complex in *L. tarentolae* (26); tRNAsynt, tRNA synthetase; PseudoU_synth, pseudouridine synthase; ECH, enoyl-CoA hydratase; GH, glycohydrolase.

Gene ID	GeneDB	Homology search (*E*-score)	Domain search (*E*-score)
Tb927.1.2990	HP	*Arabidopsis* PPR protein (1e−07)	PPR (0.000022)
Tb927.3.970	HP	Bacterial 3-hydroxyisobutyrate dehydrogenase (6e−14)	MmsB (2e−26); NAD_binding_2 (2e−21)
Tb927.4.3690[TAP_SSU4]	HP	*Campylobacter* superoxide dismutase (7e−07)	SodA (1e−08); Sod_Fe_C (3.5e−05)
Tb927.6.4930*	HP	Mammalian hypothetical protein (7e−04)	RHOD_HSP67B2 (1e−09)
Tb10.61.0690	tRNAsynt	Bacterial tRNA pseudouridine synthase (1e−08)	PseudoU_synth (7e−18)
Tb11.01.1910	HP	*Burkholderia* enoyl-CoA hydratase (1e−04)	ECH (8e−08)
Tb11.02.2710	HP	*Bos* methyltransferase 11 (3e−14)	Rsm 22 (1e−13); rRNA methylase (1e−08)
Tb927.3.2260*	HP		Ubiquitin (0.0007)
Tb927.3.5240*	HP		DUF436 (0.2); PPR (3.6)
Tb927.7.2620*	HP		Apoptosis regulator Bcl-2
Tb927.8.4860	HP		PPR (0.18)
Tb10.26.0050	HP		PPR (0.39)
Tb11.01.3710	HP		ADP-ribosyl-GH (0.002)
Tb11.02.3180*	HP		PPR

Tb927.2.4400*, Tb927.3.770*, Tb927.5.1510*[TAP_SSU5], Tb927.5.1790*, Tb927.5.3640*, Tb927.5.4040*, Tb927.6.2080*[TAP_SSU6], Tb927.6.2180*, Tb927.6.4080, Tb927.6.4560*, Tb927.6.4580*, Tb927.7.3050*, Tb927.7.3240*, Tb927.8.1430, Tb927.8.4550, Tb927.8.5200*, Tb09.160.3800*, Tb09.160.4750*, Tb09.211.2430*, Tb09.211.3000*, Tb09.211.4510*, Tb10.26.0750, Tb10.61.0190, Tb10.61.3110*, Tb10.70.3840*, Tb10.70.4220*, Tb11.01.2170*, Tb11.01.3300*, Tb11.01.7510, Tb11.02.0130*, Tb11.47.0024*

Data analysis of six tagged complexes for each subunit allowed high confidence assignment, *i.e.* at least two peptide matches with a probability of 1 in three (LSU) or in four (SSU) different TAP tags, of 77 proteins to the LSU and 56 proteins to the SSU. A control purification of the tag alone did not reveal any of those proteins thus illustrating their specific interactions with the bait proteins. Moreover none of those proteins have been described as the component of other TAP-tagged mt complexes (33, 39). Orthologs of all 49 proteins that were found in the 45 S SSU*-related ribosomal complex from *L. tarentolae* (26) were identified, although five of these did not meet our high confidence criteria (supplemental Table 4).

The *T. brucei* genome contains 41 proteins with homology to mammalian mt ribosomal proteins and bacterial ribosomal proteins of which most were annotated as hypothetical proteins. All of them were identified with high confidence in the purified ribosome except for three proteins that have borderline homology (L10 and MRPL40) or appear to encode a tRNA synthetase (MRPL39). Because the candidate proteins presented here are physically associated with affinity-purified ribosomes and they possess some level of sequence homology to prokaryotic/eukaryotic ribosomal proteins, the confidence level of their assignment as mt ribosomal proteins is further enhanced.

The number of prokaryotic homologs found in the *T. brucei* genome (9 SSU and 22 LSU homologs) is somewhat smaller than the number observed in yeast mt ribosome (16 SSU and 23 LSU homologs), mammalian mt ribosome (14 SSU and 28 LSU homologs), and *Chlamydomonas* chloroplast ribosome (19 SSU and 27 LSU homologs). Nevertheless it appears that the genome of *T. brucei* does not encode any more prokaryotic homologs of ribosomal proteins in addition to those found in this

study. Mt ribosomal protein homologs that were not found in the *T. brucei* genome and in purified mt ribosomes have sequence homologies that may have diverged beyond confident recognition, or they are absent or were potentially replaced by other proteins. Most of the *Trypanosoma* mt ribosomal proteins that have bacterial homologs also have homologs in mammalian mt ribosome, reminiscent of their common, eubacterial origin, with the exception of ribosomal proteins S8 and L29. All identified homologs of *T. brucei* mt ribosomal proteins, except for the mitochondrially encoded RPS12, are larger than their counterparts in *E. coli* and mammalian mitochondria. This may compensate for a weak structural scaffold provided by the smaller 9 and 12 S rRNAs or represent alternative functions.

We also identified 56 proteins with high confidence assignment to purified ribosomes but that have no recognizable homology outside the Kinetoplastida. This suggests that their divergence was sufficient to prevent recognition of sequence relatedness or that they are unique to kinetoplastid mitochondria. It is likely that many, and possibly all, of these proteins are highly diverged integral mt ribosomal proteins or represent the acquisition or replacement by novel proteins that reflect changes in ribosomal protein functions as has been shown for mammalian mt ribosomes (37). However, more detailed structural and functional characterization is needed to assess the specific functions of these candidate component proteins.

Several other *Trypanosoma* proteins were identified in the purified ribosomes that had no evident homology to other ribosomal proteins but had motifs suggestive of possible functions associated with mt ribosomes and rRNAs. PPR motif was identified within eight proteins of which three proteins (Tb10.70.7960, Tb927.1.2990, and Tb927.1.1160) were shown to be required for

the stabilization of 12 and 9 S rRNAs (40). The other motifs indicate GTP binding, GTPase, methyltransferase, peptidyl-prolyl isomerase, helicase activities, and chaperone function. These proteins may have roles in ribosome maturation or assembly, RNA folding, rRNA processing, protein assembly, and subunit structure stabilization similar to what has been observed in other organisms (41–49). Intriguingly helicase activity was recently identified in ribosomes that could account for its ability to translate through downstream mRNA secondary structure (50).

Moreover several ribosomal proteins have roles unrelated to translation (51). For example, we found the *T. brucei* protein homolog of yeast and bovine MRPS29 that is the death-associated protein 3 involved in apoptosis (8, 44, 52). Although the nature of apoptosis in this unicellular organism is uncertain, a role for mt ribosomes in this process cannot be disregarded because another *T. brucei* mt ribosome-associated protein (Tb927.7.2620) that we found belongs to the Bcl-2 family (53). The apparent strong association of a putative superoxide dismutase homolog in *T. brucei* mt ribosomes (*i.e.* the tagged protein pulled down the SSU) implies a possible antioxidant role, which may parallel the roles of the yeast Mrp1 and Rsm26 mt ribosomal proteins that also share similarities with proteins of the superoxide dismutase family (44).

The number of identified proteins is high, and they may not all be attributed to stable SSU and LSU particles alone. It is possible that the purified complexes are not only ribosomes but are part of a larger supercomplex with an additional function such as RNA processing, translation control, mRNA recognition, rRNA maturation, rRNA modification, or ribosome assembly as also has been suggested for the unusual ribosomal complexes in *Leishmania* (26).

This comprehensive characterization of the protein composition of *T. brucei* mt ribosomes and the associated bioinformatics provides functional designations for 29 proteins that were previously designated as hypothetical in the GeneDB database and suggests a putative role associated with protein biosynthesis for more than 100 proteins. These results expand our knowledge of the unique mt translational machinery in these early diverged parasitic protozoa. Additional studies are needed to define specific roles for individual proteins in mitochondrial protein translation; this should now be greatly facilitated by having the set of the ribosomal proteins defined.

‡ To whom correspondence should be addressed: Seattle Biomedical Research Inst., 307 Westlake Ave. N., Suite 500, Seattle, WA 98109-5219. Tel.: 206-256-7316; Fax: 206-256-7229; E-mail: ken.stuart@sbri.org.

REFERENCES

1. Matthews, D. E., Hessler, R. A., Denslow, N. D., Edwards, J. S., and O'Brien, T. W. (1982) Protein composition of the bovine mitochondrial ribosome. *J. Biol. Chem.* **257,** 8788–8794
2. Smits, P., Smeitink, J. A., van den Heuvel, L. P., Huynen, M. A., and Ettema, T. J. (2007) Reconstructing the evolution of the mitochondrial ribosomal proteome. *Nucleic Acids Res.* **35,** 4686–4703
3. Maslov, D. A., Sharma, M. R., Butler, E., Falick, A. M., Gingery, M., Agrawal, R. K., Spremulli, L. L., and Simpson, L. (2006) Isolation and characterization of mitochondrial ribosomes and ribosomal subunits from Leishmania tarentolae. *Mol. Biochem. Parasitol.* **148,** 69–78
4. Leaver, C. J., and Harmey, M. A. (1972) Isolation and characterization of mitochondrial ribosomes from higher plants. *Biochem. J.* **129,** 37P–38P
5. O'Brien, T. W. (2003) Properties of human mitochondrial ribosomes. *IUBMB Life* **55,** 505–513
6. Graack, H. R., and Wittmann-Liebold, B. (1998) Mitochondrial ribosomal proteins (MRPs) of yeast. *Biochem. J.* **329,** 433–448
7. Suzuki, T., Terasaki, M., Takemoto-Hori, C., Hanada, T., Ueda, T., Wada, A., and Watanabe, K. (2001) Proteomic analysis of the mammalian mitochondrial ribosome. Identification of protein components in the 28 S small subunit. *J. Biol. Chem.* **276,** 33181–33195
8. Suzuki, T., Terasaki, M., Takemoto-Hori, C., Hanada, T., Ueda, T., Wada, A., and Watanabe, K. (2001) Structural compensation for the deficit of rRNA with proteins in the mammalian mitochondrial ribosome. Systematic analysis of protein components of the large ribosomal subunit from mammalian mitochondria. *J. Biol. Chem.* **276,** 21724–21736
9. Koc, E. C., Burkhart, W., Blackburn, K., Moyer, M. B., Schlatzer, D. M., Moseley, A., and Spremulli, L. L. (2001) The large subunit of the mammalian mitochondrial ribosome. Analysis of the complement of ribosomal proteins present. *J. Biol. Chem.* **276,** 43958–43969
10. Cavdar, K. E., Burkhart, W., Blackburn, K., Moseley, A., and Spremulli, L. L. (2001) The small subunit of the mammalian mitochondrial ribosome. Identification of the full complement of ribosomal proteins present. *J. Biol. Chem.* **276,** 19363–19374
11. Gan, X., Arita, K., Isono, S., Kitakawa, M., Yoshino, K., Yonezawa, K., Kato, A., Inoue, H., and Isono, K. (2006) Identification and comparative analysis of the large subunit mitochondrial ribosomal proteins of Neurospora crassa. *FEMS Microbiol. Lett.* **254,** 157–164
12. Gan, X., Kitakawa, M., Yoshino, K., Oshiro, N., Yonezawa, K., and Isono, K. (2002) Tag-mediated isolation of yeast mitochondrial ribosome and mass spectrometric identification of its new components. *Eur. J. Biochem.* **269,** 5203–5214
13. Yamaguchi, K., von, K. K., and Subramanian, A. R. (2000) The plastid ribosomal proteins. Identification of all the proteins in the 30 S subunit of an organelle ribosome (chloroplast). *J. Biol. Chem.* **275,** 28455–28465
14. Yamaguchi, K., and Subramanian, A. R. (2000) The plastid ribosomal proteins. Identification of all the proteins in the 50 S subunit of an organelle ribosome (chloroplast). *J. Biol. Chem.* **275,** 28466–28482
15. O'Brien, T. W. (2002) Evolution of a protein-rich mitochondrial ribosome: implications for human genetic disease. *Gene (Amst.)* **286,** 73–79
16. Lukes, J., Hashimi, H., and Zikova, A. (2005) Unexplained complexity of the mitochondrial genome and transcriptome in kinetoplastid flagellates. *Curr. Genet.* **48,** 277–299
17. Stuart, K. D., Schnaufer, A., Ernst, N. L., and Panigrahi, A. K. (2005) Complex management: RNA editing in trypanosomes. *Trends Biochem. Sci.* **30,** 97–105
18. Horváth, A., Berry, E. A., and Maslov, D. A. (2000) Translation of the edited mRNA for cytochrome b in trypanosome mitochondria. *Science* **287,** 1639–1640
19. Horváth, A., Kingan, T. G., and Maslov, D. A. (2000) Detection of the mitochondrially encoded cytochrome *c* oxidase subunit I in the trypanosomatid protozoan *Leishmania tarentolae*. *J. Biol. Chem.* **275,** 17160–17165
20. Horvath, A., Nebohacova, M., Lukes, J., and Maslov, D. A. (2002) Unusual polypeptide synthesis in the kinetoplast-mitochondria from Leishmania tarentolae. Identification of individual de novo translation products.

J. Biol. Chem. **277,** 7222–7230

21. de la Cruz, V. F., Lake, J. A., Simpson, A. M., and Simpson, L. (1985) A minimal ribosomal RNA: sequence and secondary structure of the 9S kinetoplast ribosomal RNA from Leishmania tarentolae. *Proc. Natl. Acad. Sci. U. S. A.* **82,** 1401–1405

22. de la Cruz, V. F., Simpson, L., and Lake, J. (1985) Primary sequence and partial secondary structure of the 12S kinetoplast (mitochondrial) ribosomal RNA from Leishmania tarentolae: conservation of peptidyl-transferase structural elements. *Nucleic Acids Res.* **13,** 2337–2355

23. Sloof, P., Van den Burg, J., Voogd, A., Benne, R., Agostinelli, M., Borst, P., Gutell, R., and Noller, H. (1985) Further characterization of the extremely small mitochondrial ribosomal RNAs from trypanosomes: a detailed comparison of 9S and 12S RNAs from *Crithidia fasciculata* and *Trypanosoma brucei* with rRNAs from other organisms. *Nucleic Acids Res.* **13,** 4171–4190

24. Eperon, I. C., Janssen, J. W., Hoeijmakers, J. H., and Borst, P. (1983) The major transcripts of the kinetoplast DNA of Trypanosoma brucei are very small ribosomal RNAs. *Nucleic Acids Res.* **11,** 105–125

25. Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J., Staden, R., and Young, I. G. (1981) Sequence and organization of the human mitochondrial genome. *Nature* **290,** 457–465

26. Maslov, D. A., Spremulli, L. L., Sharma, M. R., Bhargava, K., Grasso, D., Falick, A. M., Agrawal, R. K., Parker, C. E., and Simpson, L. (2007) Proteomics and electron microscopic characterization of the unusual mitochondrial ribosome-related 45S complex in Leishmania tarentolae. *Mol. Biochem. Parasitol.* **152,** 203–212

27. Jensen, B. C., Kifer, C. T., Brekken, D. L., Randall, A. C., Wang, Q., Drees, B. L., and Parsons, M. (2007) Characterization of protein kinase CK2 from Trypanosoma brucei. *Mol. Biochem. Parasitol.* **151,** 28–40

28. Panigrahi, A. K., Schnaufer, A., Ernst, N. L., Wang, B., Carmean, N., Salavati, R., and Stuart, K. (2003) Identification of novel components of *Trypanosoma brucei* editosomes. *RNA* **9,** 484–492

29. Wirtz, E., Leal, S., Ochatt, C., and Cross, G. A. M. (1999) A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei. Mol. Biochem. Parasitol.* **99,** 89–101

30. Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Seraphin, B. (2001) The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods* **24,** 218–229

31. Schnaufer, A., Ernst, N., O'Rear, J., Salavati, R., and Stuart, K. (2003) Separate insertion and deletion sub-complexes of the *Trypanosoma brucei* RNA editing complex. *Mol. Cell* **12,** 307–319

32. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999) A generic protein purification method for protein complex characterization and proteome exploration. *Nat. Biotechnol.* **17,** 1030–1032

33. Panigrahi, A. K., Zikova, A., Dalley, R. A., Acestor, N., Ogata, Y., Anupama, A., Myler, P. J., and Stuart, K. D. (2008) Mitochondrial complexes in trypanosoma brucei: a novel complex and a unique oxidoreductase complex. *Mol. Cell. Proteomics* **7,** 534–545

34. Berriman, M., Ghedin, E., Hertz-Fowler, C., Blandin, G., Lennard, N. J., Bartholomeu, D., Renauld, H. J., Caler, E., Hamlin, N., Haas, B., Harris, B. R., Hannick, L., Barrell, B., Donelson, J., Hall, N., Fraser, C. M., Melville, S. E., El-Sayed, N., Böhme, U. C., Shallom, J., Aslett, M., Hou, L., Atkin, B., Barron, A. J., Bringaud, F., Brooks, K., Cherevach, I., Chillingworth, T., Churcher, C., Clark, L. N., Corton, C. H., Cronin, A., Davies, R., Doggett, J., Djikeng, A., Feldblyum, T., Fraser, A., Goodhead, I., Hance, Z., Harper, A. D., Hauser, H., Hostetler, J., Jagels, K., Johnson, D., Johnson, J., Jones, C., Kerhornou, A., Koo, H., Larke, N., Larkin, C., Leech, V., Line, A., MacLeod, A., Mooney, P., Moule, S., Mungall, K., Norbertczak, H., Ormond, D., Pai, G., Peterson, J., Quail, M. A., Rajandream, M. A., Reitter, C., Sanders, M., Schobel, S., Sharp, S., Simmonds, M., Simpson, A. J., Tallon, L., Turner, C. M., Tait, A., Tivey, A., Van Aken, S., Walker, D., Wanless, D., White, B., White, O., Whitehead, S., Wortman, J., Barry, J. D., Fairlamb, A. H., Field, M. C., Gull, K.,

Landfear, S., Marcello, L., Martin, D. M., Opperdoes, F., Ullu, E., Whickstead, B., Alsmark, C., Arrowsmith, C., Carrington, M., Embley, T. M., Ivens, A., Lord, A., Morgan, G. M., Peacock, C. S., Rabbinowitsch, E., Salzberg, S., Wang, S., Woodward, J., and Adams, M. D. (2005) The genome of the African trypanosome, *Trypanosoma brucei. Science* **309,** 416–422

35. Keller, A., Nesvizhskii, A. I., Kolker, E., and Aebersold, R. (2002) Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.* **74,** 5383–5392

36. Nesvizhskii, A. I., Keller, A., Kolker, E., and Aebersold, R. (2003) A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* **75,** 4646–4658

37. Sharma, M. R., Koc, E. C., Datta, P. P., Booth, T. M., Spremulli, L. L., and Agrawal, R. K. (2003) Structure of the mammalian mitochondrial ribosome reveals an expanded functional role for its component proteins. *Cell* **115,** 97–108

38. Kitakawa, M., Graack, H. R., Grohmann, L., Goldschmidt-Reisin, S., Herfurth, E., Wittmann-Liebold, B., Nishimura, T., and Isono, K. (1997) Identification and characterization of the genes for mitochondrial ribosomal proteins of Saccharomyces cerevisiae. *Eur. J. Biochem.* **245,** 449–456

39. Panigrahi, A. K., Ernst, N. L., Domingo, G. J., Fleck, M., Salavati, R., and Stuart, K. D. (2006) Compositionally and functionally distinct editosomes in *Trypanosoma brucei. RNA* **12,** 1038–1049

40. Pusnik, M., Small, I., Read, L. K., Fabbro, T., and Schneider, A. (2007) Pentatricopeptide repeat proteins in Trypanosoma brucei function in mitochondrial ribosomes. *Mol. Cell. Biol.* **27,** 6876–6888

41. Denslow, N. D., Anders, J. C., and O'Brien, T. W. (1991) Bovine mitochondrial ribosomes possess a high affinity binding site for guanine nucleotides. *J. Biol. Chem.* **266,** 9586–9590

42. Scott, J. M., Ju, J., Mitchell, T., and Haldenwang, W. G. (2000) The Bacillus subtilis GTP binding protein obg and regulators of the sigma(B) stress response transcription factor cofractionate with ribosomes. *J. Bacteriol.* **182,** 2771–2777

43. Sato, A., Kobayashi, G., Hayashi, H., Yoshida, H., Wada, A., Maeda, M., Hiraga, S., Takeyasu, K., and Wada, C. (2005) The GTP binding protein Obg homolog ObgE is involved in ribosome maturation. *Genes Cells* **10,** 393–408

44. Saveanu, C., Fromont-Racine, M., Harington, A., Ricard, F., Namane, A., and Jacquier, A. (2001) Identification of 12 new yeast mitochondrial ribosomal proteins including 6 that have no prokaryotic homologues. *J. Biol. Chem.* **276,** 15861–15867

45. Conrad, J., Sun, D., Englund, N., and Ofengand, J. (1998) The rluC gene of Escherichia coli codes for a pseudouridine synthase that is solely responsible for synthesis of pseudouridine at positions 955, 2504, and 2580 in 23 S ribosomal RNA. *J. Biol. Chem.* **273,** 18562–18566

46. Dresios, J., Panopoulos, P., and Synetos, D. (2006) Eukaryotic ribosomal proteins lacking a eubacterial counterpart: important players in ribosomal function. *Mol. Microbiol.* **59,** 1651–1663

47. Fatica, A., and Tollervey, D. (2002) Making ribosomes. *Curr. Opin. Cell Biol.* **14,** 313–318

48. Wilson, D. N., and Nierhaus, K. H. (2007) The weird and wonderful world of bacterial ribosome regulation. *Crit. Rev. Biochem. Mol. Biol.* **42,** 187–219

49. Wilson, D. N., and Nierhaus, K. H. (2005) Ribosomal proteins in the spotlight. *Crit. Rev. Biochem. Mol. Biol.* **40,** 243–267

50. Takyar, S., Hickerson, R. P., and Noller, H. F. (2005) mRNA helicase activity of the ribosome. *Cell* **120,** 49–58

51. Brodersen, D. E., and Nissen, P. (2005) The social life of ribosomal proteins. *FEBS J.* **272,** 2098–2108

52. Cavdar, K. E., Ranasinghe, A., Burkhart, W., Blackburn, K., Koc, H., Moseley, A., and Spremulli, L. L. (2001) A new face on apoptosis: death-associated protein 3 and PDCD9 are mitochondrial ribosomal proteins. *FEBS Lett.* **492,** 166–170

53. Reed, J. C., Zha, H., Aime-Sempe, C., Takayama, S., and Wang, H. G. (1996) Structure-function analysis of Bcl-2 family proteins. Regulators of programmed cell death. *Adv. Exp. Med. Biol.* **406,** 99–112